IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of : Shoichi OZAKI et al.
Serial No.: 09/214,881
Filed: June 7, 1999
For: DIAGNOSTIC DRUGS FOR AUTOIMMUNE DISEASE

DECLARATION UNDER 37 CFR § 1.132

Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Fumio OSAKADA, declare as follows:

1. I, Fumio OSAKADA, have following postal address: C/O KANEKA CORPORATION, 2-4, Nakanoshima 3-chome, Kita-ku, Osaka-shi Osaka 530 Japan.

2. I received a Doctor's degree from the Graduate School of Agriculture, University of Tsukuba in March 23, 1987.

3. I have been employed by KANEKA CORPORATION since 1987. I have been engaged in research of diagnostic drugs for autoimmune diseases. I am one of the inventors of the above-identified application and I am fully familiar with the subject matter thereof.

4. Based on our experience with regard to the subject matter of the above-identified application, the following ex-

periments were conducted to demonstrate that i)fragments of HMG-1 or HMG-2 react with sera from an autoimmune disease patient, and can be used for diagnosing an autoimmune disease; and ii)a polypeptide having an amino acid sequence homology of 90% or more with HMG-1, or a polypeptide having an amino acid sequence homology of 80% or more with HMG-2 can react with an antibody from an antoimmune disease patient.

Together with this declaration, amendments of claims will be submitted in the United States Patent and Trademark Office, and I, therefore, refer to the amended claims in the following description.

Experiments.

The inventors examined the reactivity of HMG-1 and HMG-2 fragment peptides with the anti-HMG-1 and anti-HMG-2 antibodies in autoimmune disease patients.

Figure 1 illustrates two fragment peptides used in the experiments, which are derived from porcine HMG-1 and porcine HMG-2, respectively. The peptides were expressed in recombinant E. coli, and purified using alkyl Superlose.

(1) Figures 2, 3 and 4 illustrate the results of Western blotting in which sera from ulcerative colitis patients, which were positive for anti-HMG-1 and anti-HMG-2 antibodies in ELISA using HMG-1 and HMG-2 fragment peptides as antigens, were used as probes. Western blotting was performed as described in the specification of the present application. Black bands in figures 2, 3 and 4 indicate peptide fragments which reacted to the antibodies. The name of the examined fragment peptide is indicated in the vicinity of each black band.

As can be seen from figure 2, regarding HMG-1 fragment peptides, a serum of an ulcerative colitis patient i) reacted predominantly with the AIB fragment; ii) did not react with the A fragment; and iii) reacted with the AI fragment at a low level. As such, this indicates that an epitope for the serum exists after the 76-th position in the amino acid sequence of porcine HMG-1. Regarding HMG-2 fragment peptides, since the serum strongly reacted with the AIB and the B fragments, this indicates that an epitope for the serum exists between the 88-th and 164-th amino acids in the HMG-2 peptide. The serum also reacted with the A and AI fragments, which may indicate the presence of an antibody recognizing a conformation of HMG-2 peptides or a distinct antibody reacting with the A and AI fragments.

As can be seen from figure 3, regarding HMG-1 fragment peptides, since the serum reacted strongly with the A fragment, this indicates that an epitope for the serum exists in the amino acid sequence ranging from the 1-st to the 76-th amino acid. Regarding HMG-2 fragment peptides, although the serum reacted with the HMG-2 peptide itself, significant binding between the serum and any fragment could not be observed. As such, it is considered that i) epitope exists in the amino acid sequence ranging from the 164-th to the 209-th amino acids in HMG-2 fragment, which were not examined in this study, or ii) the serum recognizes the conformation of the full-length HMG-2 peptide. The serum did not react with the Bj fragment of HMG-1, or the B and Bj fragments of HMG-2.

As can be seen from figure 4, regarding HMG-1 fragment peptides, the serum reacted equally with the A, AI and AIB fragments. As such, it is considered that an epitope for the serum exists in the A fragment which contains the

amino acid sequence ranging from the 1-st to the 76-th amino acids. Similarly, it is considered that an epitope for the serum exists in the A fragment. The serum did not react with the Bj fragment of HMG-1, or the B and Bj fragments of HMG-2.

The results of figures 2, 3 and 4 are summarized in figure 5. In summary, according to the results indicated in figures 2, 3 and 4, it is indicated that anti-HMG-1 antibodies and anti-HMG-2 antibodies recognize different epitopes depending on the individual patient.

(2) Figures 6 and 7 summarize the epitope analysis of HMG-1 and HMG-2 peptides in autoimmune disease. As described in the above (1), Western blotting was performed in which sera from autoimmune patients having anti-HMG-1 and anti-HMG-2 antibodies were used as probes and antigens, respectively. The graphs in the upper row of figures 6 and 7 indicate the result of the analysis. The tables in the lower row of figures 6 and 7 indicate the constitutions of the mixtures of peptide fragments. + denotes the presence of the fragment. - denotes the absence of the fragment. RA, SLE, SSc, SS, AIH, PBC, and UC stand for rheumatoid arthritis, systemic lupus erythematosus, Sjögren's syndrome, scleroderma, autoimmune hepatitis, primary biliary cirrhosis, and ulcerative colitis.

As can be seen from figure 6 (HMG-1 fragment), the sera which reacted specifically with the AIB fragment without reacting with the A, AI or Bj fragments is 100% in RA, 71% in SSc, 100% in SS, 100% in AIH, 83% in PBC, and 80% in UC, respectively. As for SLE, 40% of sera reacted specifically with the AIB fragment, and 50% of sera reacted with the mixture of the AI and AIB fragments.

As can be seen from figure 7 (HMG-2 fragment), which is similar to the results of HMG-1 fragments, most sera reacted specifically with the AIB fragment as follows: 72% in SLE, 67% in SSc, 100% in SS, 88% in AIH, and 75% in PBC. In RA and UC, 50% of sera reacted specifically with the AIB fragment, the remainder are antibodies which recognize another portion of the HMG-2 peptide.

According to the results indicated in figures 6 and 7, it was indicated that both AIB fragment peptides of HMG-1 and HMG-2 peptide can be used in diagnosing the above autoimmune diseases instead of HMG-1 and HMG-2 peptides.

In the meantime, there is a sequence homology of 79% between porcine HMG-1 and porcine HMG-2 used in the above experiments. As indicated in figures 2, 3, and 4, serum of an autoimmune patient reacts with both HMG-1 and HMG-2. That is to say, in the event that polypeptides having a sequence homology of 79 % with HMG-1 or HMG-2, serum of an autoimmune patient reacts with both polypeptide.

The results of searching a sequence homology between porcine HMG-1 and porcine HMG-2 are attached herewith. In the search, Query is porcine HMG-1, and subject is porcine HMG-2. The search was conducted at Web site of U.S. National Center for Biotechnology Information (NCBI), the Official site of NIH. In the search, since both HMG-1 and HMG-2 are translated from DNA, both polypeptides initiate at methionine. As a result, they have an additional amino acid compared to the original mature HMG polypeptides. That is, in the searching, HMG-1 has 215 amino acids instead of 214 amino acids, and HMG-2 has 210 amino acids instead of 209 amino acids. However, there is a sequence homology of 79% in both cases and the result was not affected.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Executed on January 21, 2002.

Fumio Osakada

(Fumio OSAKADA)

Fig.1

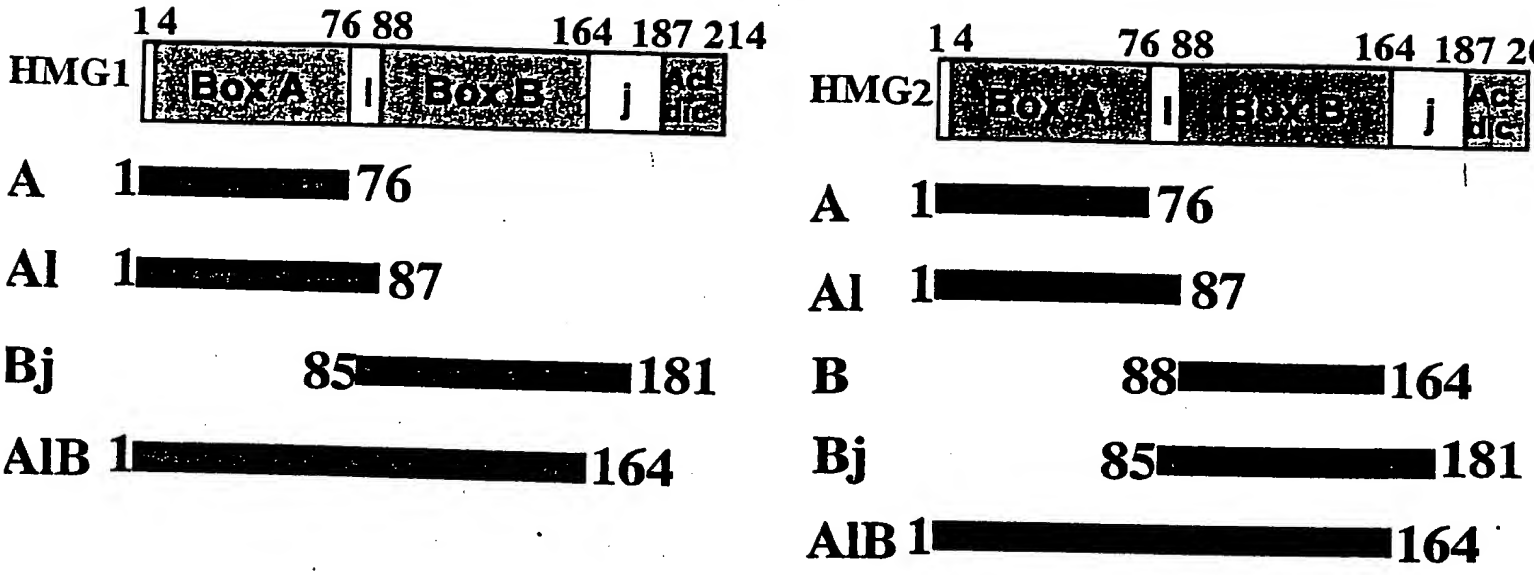


Fig.2

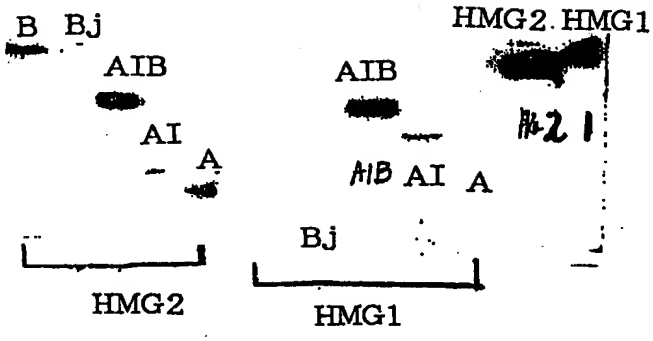


Fig.3

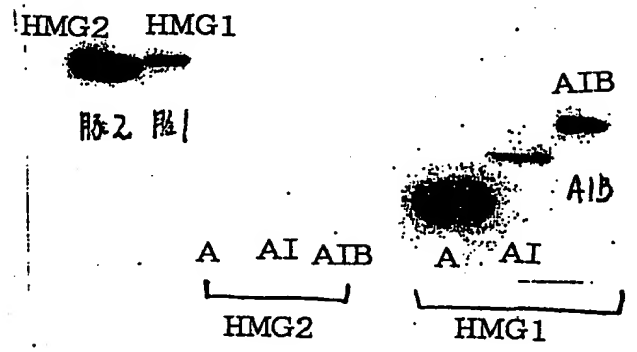


Fig.4

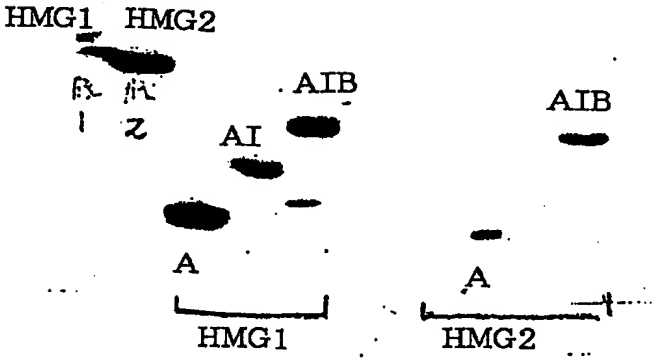
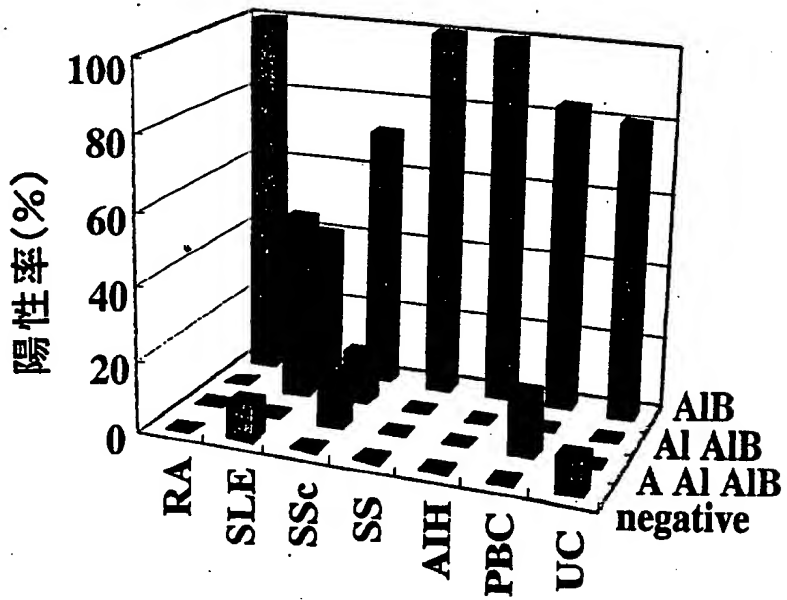


Fig.5 Epitope recognized by sera from ulcerative colitis pateints.

HMG antigen	Peptide fragment containing the estimated epitope		
	Figure 2	Figure 3	Figure 4
HMG-1	76-164	1-76	1-76
HMG-2	1-76 88-164 1-164	1-209 165-209	1-164

Fig.6 Epitope analysis of HMG-1 peptide in autoimmune diseases.



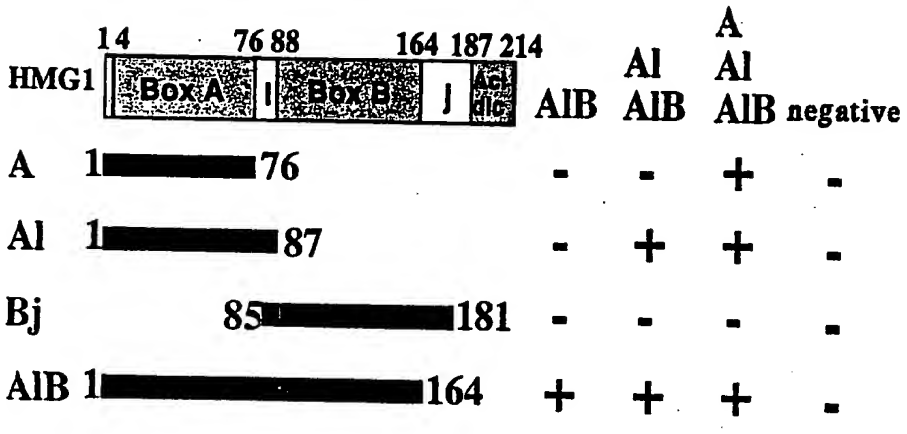
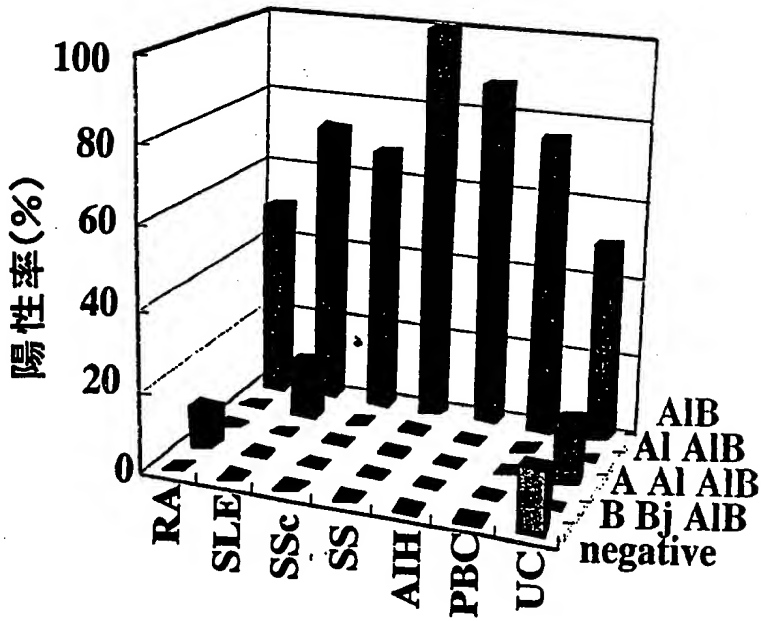
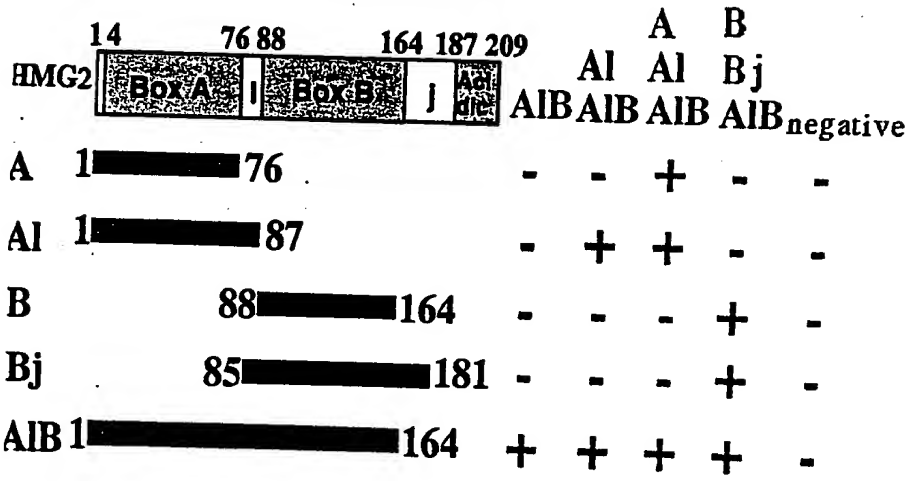


Fig.7 Epitope analysis of HMG-2 peptide in autoimmune diseases.





NCBI **Blast 2 Sequences results**

PubMed Entrez BLAST OMIM Taxonomy Structure

BLAST 2 SEQUENCES RESULTS VERSION BLASTP 2.2.2 [Dec-14-2001]

Matrix: BLOSUM62 gap open: 11 gap extension: 1
x_dropoff: 50 expect: 10.00 wordsize: 3 Filter Align

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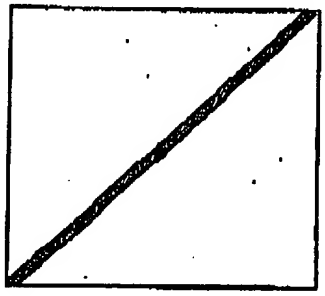
Sequence 2 gi|108366 nonhistone chromosomal protein HMG-2 - pig Length 210 (1 .. 210)



NOTE:The statistics (bitscore and expect value) is calculated based on the size of nr database

Score = 357 bits (917), Expect = 2e-98
Identities = 167/210 (79%), Positives = 189/210 (89%)

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Sbjct:                          1  MGKGDPNKPRGKMSSYAFFVQTCREEHKKKHPDSSVNFAEFSKKCSERWKTMSAKEKSKF 60
HMG box homology #label HMG1 6     ************************************************************

Query:                         61  EDMAKADKARYEREMKTYIPPKGETKKKFKDPNAPKRPPSAFFLFCSEYRPKIKGEHPGL 120
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Sbjct:                         61  EDMAKSDKARYDREMKNYVPPKGDKKGKKKDPNAPKRPPSAFFLFCSEHRPKIKSEHPGL 120
HMG box homology #label HMG2 92                              ******************************
HMG box homology #label HMG1 61  ************************

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Sbjct:                        121  SIGDTAKKLGEMWSEQSAKDKQPYEQKAAKLKEKYEKDIAAYRAKGKGEAGKKGPGRPTG 180
HMG box homology #label HMG2 121 ************************************************

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CPU time: 0.05 user secs. 0.02 sys. secs 0.07 total secs.

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Lambda     K      H
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Gapped
Lambda     K      H
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Matrix: BLOSUM62
Gap Penalties: Existence: 11, Extension: 1
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Number of extensions: 128
Number of successful extensions: 39
Number of sequences better than 10.0: 1
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Number of HSP's successfully gapped in prelim test: 0
Number of HSP's that attempted gapping in pr lim test: 0
Number of HSP's gapped (non-prelim): 6
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length of database: 265,492,265
effective HSP length: 112
effective length of query: 98
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A: 40
X1: 17 (7.4 bits)
X2: 129 (49.7 bits)
X3: 129 (49.7 bits)
S1: 43 (21.8 bits)
S2: 67 (30.4 bits)